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13. SUPPLEMENTARY NOTES

14. ABSTRACT

GFP plasmids were constructed and used for transfection of blood and sporozoite stages of *P. vivax*. GFP transformed parasites were observed when transfection was performed using blood stage parasites. Transfection of GFP plasmids into liver cells could enhance plasmid uptake of the sporozoites after invasion of the liver cells. Optimization of transfection for the GFP plasmids to blood and sporozoite stages need to be further optimized. Partial proteomic of sporozoite were studied and some parasite proteins were found to be up-regulated at this stage. These proteins could be used as markers to follow the parasite development in the liver cells *in vitro*.

15. SUBJECT TERMS
GFP-transfection, mosquito stages of P. vivax, liver stage parasite of P. vivax

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INTRODUCTION.

This research aims to study gene and protein expression of Plasmodium vivax liver stage. We propose to transfect the parasites with green fluorescent expressing plasmids (GFP-plasmids) at different developmental stage to identify the liver stage parasites develop in vitro in human liver hepatocyte cell line, HC04. Since continuous culture of P. vivax is not feasible yet we will introduce GFP plasmid to different stages of P. vivax by (a) transfecting blood stage with GFP plasmid and culture for 24 hr before feeding to the mosquitoes to obtain GFP transfected sporozoites, (b) transfecting sporozoites with GFP plasmid and (c) transfecting HC04 liver cells with GFP plasmid thus the parasites will uptake this plasmid during development in the liver cells. Sporozoites will be alternatively tagged by TAT-GFP before invasion and development in liver cells. At different time intervals GFP expressing infected liver cells will be separated from non-infected cells by fluorescence activated cell sorting (FACS). The cell fractions will be collected for further gene and protein analysis. Total RNA and protein will be prepared from the infected cells. A cDNA library will be constructed using a PCR based amplification procedure. This technique is very suitable for cDNA library construction from limited amount of samples. Such a library will be used in expressed sequence tag (EST) analysis to study the transcriptome of the liver stage parasite. Protein prepared from infected cell lysate will be used for SDS-PAGE and further analyzed by using nano-capillary reverse-phase LC-MS/MS analysis system. Parasite proteins will be catalogued and compared to the EST study. We anticipate that this study will elucidate quantitative gene expression profile of the malaria parasite and reveal novel genes and proteins for vaccine and drug targets.

BODY.

Progress for the reporting period:

1. GFP-Plasmid construction:

Plasmids comprising of promoter (EF1 or HSP86), selectable marker (hDHFR) and GFP reporter were constructed and used for transfection experiments. These plasmids were tested for ability to express GFP protein in malaria parasites by transfection to *P. falciparum* cultured *in vitro*.

2. Transfection of GFP plasmids to different stages of *P. vivax*:

2.1) Transfection of gametocyte stage using reagents and programs provided by the AMAXA company. Solutions and programs that could transform *P. vivax* gametocytes to produce GFP gamete or ookinete have been shown in table1. Although GFP parasites were observed in blood meal after feeding but very few oocysts were found

positive for GFP. This depended on gametocyte number and infectivity as well as the efficiency of transfection procedures. Different protocols need to be tries with more blood samples to optimize the transfection process.

2.2) Transfection of *Plasmodium vivax* sporozoites: Sporozoites were harvested from salivary gland and prepared for transfection using nucleofector V solution with the program of A-020, T-020, T-030, X-001, X-005, L-029, D-023. The mixture of each program was prepared as described below:

	Amount
Sporozoite	60,000 parasites
Nucleofector V solution	100 µl
piggyBAC- H86G (1µg/µl)	5 µl
pHTH (1µg/µl)	5 µl

After transfection the sporozoites were added to hepatocyte cell line cultured in 96 well plate and the culture were incubated at 37°C, 5% CO2 for 4 hrs. Medium in each well was discarded after 4 hrs. Fresh medium was added to each well and the culture plate was kept at 37°C, 5% CO2 for another 48 hrs. None of GFP transformed parasites were observed under inverted fluorescent microscope by this method.

2.3) Transfection of *P. vivax* sporozoite by lipofection: GFP plasmid was transfected into liver cells, HC04, by lipofection methods below:

2.3.1) Procedure for direct transfection: Fifty thousand sporozoites harvested from infected mosquitoes were incubated with 200 µl of transfection solutions for 20 minutes at room temperature prior to inoculate into HC-04 cells cultured in 48-well plate. The culture plate was then incubated for 4 hrs at 370C with 5% CO2 to allow the parasite invade cells. Fresh culture medium was replaced into each well and the culture plate was kept at 370C with 5% CO2 for 4 days. The transformed parasites were observed under fluorescent microscope.

2.3.2) Procedure for indirect transfection (pre-loading sporozoites): The transfection solutions were incubated with HC-04 cells cultured in 48-well plate for 2hrs prior to sporozoites inoculation. Fifty thousand sporozoites were inoculated into each well. The culture plate was incubated for 4 hrs at 370C with 5% CO2 to allow the parasite invade cells. Fresh culture medium was replaced into each well and the culture plate was kept at 370C with 5% CO2 for 4 days. The transformed parasites were observed under fluorescent microscope.

2.3.3) Procedure for indirect transfection (post-loading sporozoites): Fifty thousand sporozoites harvested from infected mosquitoes were inoculated into HC-04 cells cultured in 48-well plate. The culture plate was incubated for 4 hrs at 370C with 5% CO2 to allow the parasite invade cells. The cells were then transfected with transfection solutions prepared and incubated for 2 hrs. Fresh culture medium was replaced into each well and the culture plate was kept at 370C with 5% CO2 for 4 days. The transformed parasites were observed under fluorescent microscope.

Results from transfection of GFP plasmids to *P. vivax* sporozoites showed that direct transfection of sporozoites was unsuccessful. Conditions might need to be altered, such as greater incubation time (more than 20 minutes). When *P. falciparum* was used as control, pre-loading hepatocytes with pPfHSP60-GFP (with HSP86 promoter) plasmid by lipofection yielded small number of GFP expressing cells. Transfection post-invasion also did not produce GFP parasites but this experiment needs to be repeated since it was only attempted once. *P. vivax* sporozoites did not appear to be as amenable to transfection as *P. falciparum* in control experiment. This should be further tested at day 2 post-transfection. Transfecting preload-sporozoite invasion could potentially inhibit subsequent invasion of the cells by sporozoites (e.g. by altering/blocking surface receptors) Further testing of different time-points of lipofection might still be worthwhile. For instance, transfecting intracellular liver parasites at a later stage of development might increase the GFP expression because the amount of lipid uptake by exoerythrocytic forms would be expected to be substantial at the developing stage.

3. Production of sporozoite and preparation for transcriptome and proteomic analysis:

Sporozoites harvested from salivary gland, haemolymph and mature oocysts were prepared for proteomic study. Sporozoite total protein was measured based on Lawry method. 50 ug of sporozoite protein was run on 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G250. Thirteen bands were cut and collected from SDS-PAGE. All bands were in-gel digested and analyzed by tandem mass spectrometer (LC/MS/MS). The peaks of samples has been generated from the original data file and searched against the *P. vivax* database combined with mosquito database using ProteinLynx program.

For salivary gland sporozoite proteomic study, the results showed that most detected peptides were from mosquitoes (more than 65 %). Only ~20% of peptides were from *Plasmodium spp*. Some peptide searches were shown in table 2 and up-regulated protein expression of sporozoites were listed in table3. Data analysis has not been completed yet due to more time required because the complete genome of *P. vivax* has not been published yet

KEY RESEARCH ACCOMPLISHMENTS

- Human use protocol required for this study was approved.
- GFP plasmids were constructed and used for transfection of different stages of parasites.
- Different transfection methods have been tried. Some transfection procedures yielded GFP transformed parasites.
- Partial proteomic and gene expression of *P. vivax* sporozoites were reported.

REPORTABLE OUTCOMES. There are no reportable outcomes for this reporting period.

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CONCLUSION.

It is possible to generate GFP expressing parasites when transfection by AMAXA protocols using *P. vivax* blood stage. Transfection of GFP plasmid into liver cells, HC04, before or after sporozoites invasion could potentially induced plasmid uptake of the sporozoites. However experimental conditions need to be optimized in to increase number of GFP parasites. Proteomic analysis of sporozoites indicated that some genes have been up-regulated. This information is useful and can be selected as markers to follow the development of live stage parasites.

SUPPORTING DATA.

Table 1 Transfection of *P. vivax* gametocyte using different Nucleofector solutions and programs.

Nucleofector® solution	Program that produce GFP parasites
L	A020
	D023
	L029
	T030
	X001
	X005
V	A020
	D023
	L029
	T020
Parasite kit 1	T020
	T030
	X001
Parasite Kit 2	A020
	U33

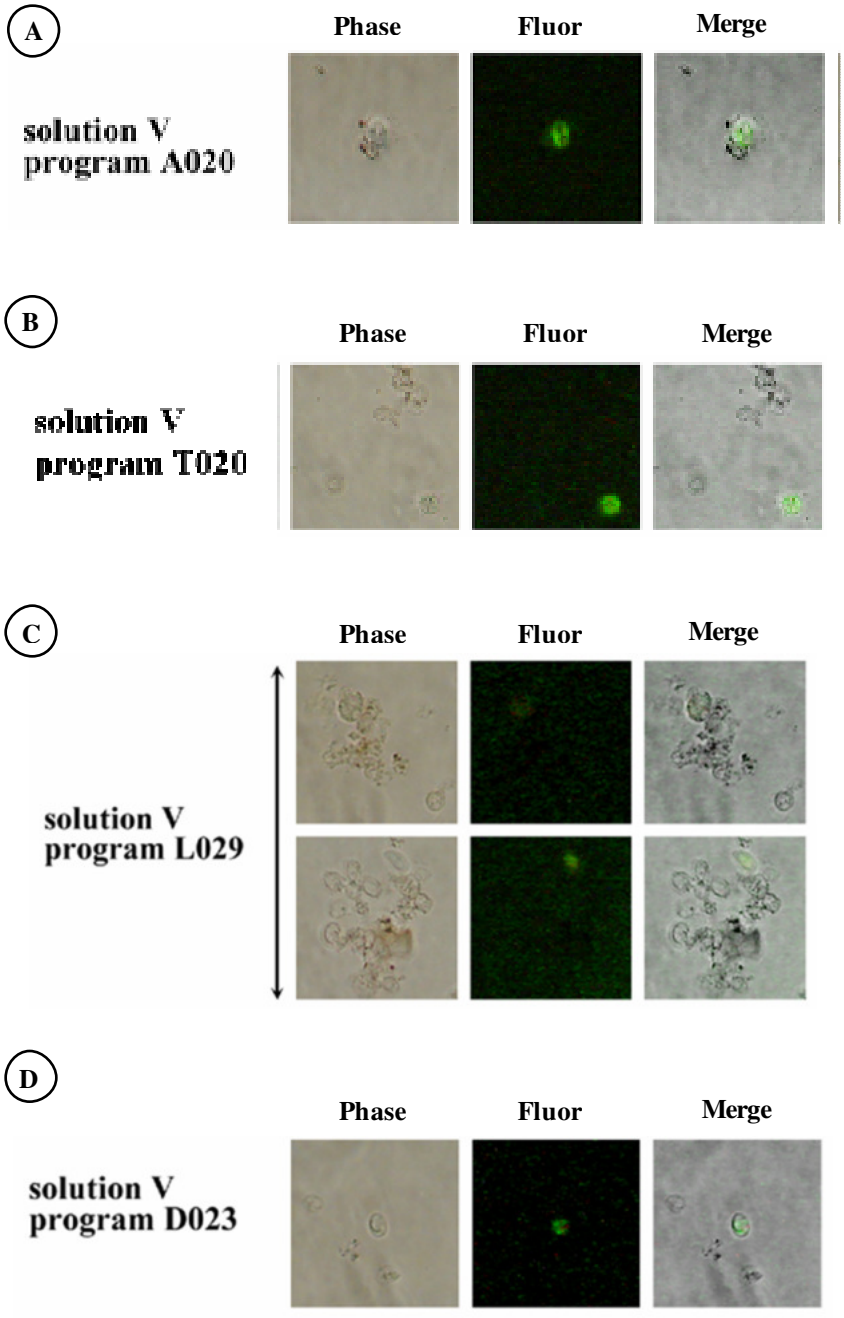

Figure 1 Images of *P. vivax* gametocyte after transfection with different solutions and conditions, and then cultured for 24 hour. The gametocytes were observed with immunofluorescent microscope using both phase contrast and blue filter. Phase: phase contrast, Fluor: fluorescence and Merge: combined phase contrast and IFA images.

Table 2: Some peptides matched to *P. vivax* database

Gene accession no.	**Protein/peptide description**
>Pv123745	endoplasmin precursor, putative
>Pv083030	myosin A, putative
>Pv099315	78 kDa glucose-regulated protein precursor (GRP 78), putative
>Pv100735	ATP synthase beta chain, mitochondrial precursor, putative
>Pv090155	tubulin alpha chain, putative
>Pv098630	tubulin alpha chain, putative
>Pv099320	acid phosphatase, putative
>Pv099380	PDI-like protein, putative
>Pv092850	small GTPase Rab6, putative
>Pv080610	small GTPase Rab1A, putative
>Pv080550	small GTPase Rab1, putative
>Pv113665	histone H3, putative
>Pv114020	histone H3, putative
>Pv123510	S4, putative

Table 3: Preliminary list of up-regulated protein expression in *P. vivax* sporozoites:

1- Pv001980_0:hypothetical protein
2- Pv123510_0:S4, putative"
3- Pv085600_1:hypothetical protein, conserved"
4- Pv096410_3:cysteine repeat modular protein 2 PbCRM2, putative"
5- Pv092585_1:adrenodoxin reductase, putative"
6- Pv096410_1:cysteine repeat modular protein 2 PbCRM2, putative"
7- Pv122065_0:heat shock protein, putative"
7- Pv088230_0:glycosyltransferase, putative"
8- Pv092275_1:apical merozoite antigen 1
9- Pv084895_0:ribosome biogenesis protein Nop10, putative"
10- Pv123575_0:thrombospondin-related protein 3 precursor, putative"
11- Pv099005_4:cysteine repeat modular protein, putative"
12- Pv091345_0:dynein light chain type 2, putative"
13- Pv082735_1:sporozoite surface protein 2
14- Pv101125_0:GTP-ase activating protein for Arf containing protein
15- Pv082415_1:adenosine/AMP deaminase, putative"
16- Pv114070_1:N-acetylglucosamine transferase, putative"
17- Pv081395_4:serine/threonine protein kinase, putative"
18- Pv000985_2:Sec24-related protein, putative"
19- Pv123050_1:phospholipase DDHD1, putative"
20- Pv116660_1:Micro-fibrillar-associated protein 1 C-terminus domain containing
21- Pv101435_2:DNA repair protein rhp16, putative"
22- Pv101075_0:vacuolar sorting protein SNF7, putative"
23- Pv097985_3:guanidine nucleotide exchange factor, putative"
24- Pv111140_0:protein phosphatase 1, regulatory (inhibitor) subunit, putative"
25- Pv080240_1:protein phosphatase 2c, putative"
26- Pv118455_1:clathrin coat assembly protein AP50, putative"
27- Pv081395_2:serine/threonine protein kinase, putative"
28- Pv101240_1:rac-beta serine/threonine protein kinase, putative"
29- Pv085315_1:PP1-like protein serine/threonine phosphatase, putative"
30-Pv095440_0:hypothetical protein